# The Tumoricidal Properties of Inflammatory Tissue Macrophages and Multinucleate Giant Cells

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**Peritoneal exudate cells from C3H/HeN mice infected with bacille Calmette Guérin (BCG) and subcutaneous inflammatory macrophages from uninfected mice exhibit spontaneous cytotoxicity for tumor cells *in vitro*, but their tumoricidal activity can be increased by incubation *in vitro* with lymphokines released by mitogen- or antigen-stimulated lymphocytes. Inflammatory macrophages from these sites are only susceptible to activation *in vitro* by lymphokines for a short period (< 4 days) following their initial emigration from the circulation to the site of inflammation. The expression of tumoricidal activity by activated macrophages is similarly short-lived (< 4 days). Once the tumoricidal state is lost it cannot be restored by further incubation with lymphokines *in vitro*. Fusion of macrophages to form multinucleate giant cells (MGCs) accompanies the loss of tumoricidal activity and the onset of resistance to activation by lymphokines, but the fusion process is not responsible for these changes, since unfused macrophages are similarly affected. Activation and acquisition of tumoricidal properties is confined to young macrophages recruited from the circulation during acute inflammation. Older macrophages and MGCs in chronic inflammatory lesions in which recruitment of new macrophages has ceased are nontumoricidal and are refractory to activation by lymphokines *in vitro*. These findings are discussed in relation to the efficiency of macrophage-mediated destruction of tumors *in vivo* and the amplification of macrophage antitumor activity by immunotherapeutic agents. (Am J Pathol 96:595–610, 1979)**

AN IMPORTANT FEATURE of host defense against infection and neoplastic disease involves activation of cells of the macrophage–histiocyte series to become cytotoxic against microorganisms and neoplastic cells.[1-3] Noncytotoxic macrophages can be rendered tumoricidal by agents that act directly or indirectly on macrophages. Direct activation occurs when macrophages interact with various bacterial products, pyran copolymers, and double-stranded RNA and after chronic infection of these cells with intracellular bacteria, protozoa, or nematode parasites.[4,5] Indirect activation involves the interaction of macrophages with soluble mediators (lymphokines) released by antigen- or mitogen-stimulated lymphocytes.[4,5] The soluble lymphocyte mediator responsible for inducing macrophage activation is referred to as macrophage-activating factor (MAF), and MAF-treated macrophages acquire the ability to recognize and nonspecifically destroy syngeneic, allogeneic, and xenogeneic neoplastic cells

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both *in vitro* and *in vivo* while leaving nonneoplastic cells unharmed.[4,5] Inflammation represents the major mechanism by which indirect activation of macrophages occurs *in vivo*. The creation of inflammatory conditions that are conducive to the recruitment and the activation of macrophages may therefore be of considerable importance in ensuring effective macrophage-mediated destruction of tumors *in vivo*.

There is increasing evidence that activation of macrophages is a complex phenomenon and that different features of the activated state can be expressed independently of others.[4,5] This evidence suggests that activation is not an all-or-none event but is a multistep process in which the properties displayed by an activated macrophage will depend on the nature of the activating stimulus. This concept is relevant to the role of inflammation in activating macrophages, since differences in the inflammatory stimulus and/or duration of inflammation (acute versus chronic) might influence the extent of macrophage activation and their antitumor activity. This question has been examined by a study of the ability of macrophages from acute and chronic inflammatory lesions to destroy tumor cells *in vitro*. These experiments indicate that the susceptibility of inflammatory macrophages to activation by lymphokines and the expression of tumoricidal properties by activated macrophages are short-lived functions. Equally important, following decay of their tumoricidal properties, inflammatory macrophages cannot be reactivated by lymphokines. These findings suggest that maintenance of effective macrophage-mediated cytotoxicity against tumor cells *in vivo* will require a constant influx of new, lymphokine-sensitive macrophages from the circulation to offset the rapid decay of tumoricidal activity in previously recruited macrophages.

## Materials and Methods

### Animals

Adult C3H mice were obtained from the West Seneca Animal Breeding Facility of Roswell Park Memorial Institute. Routine serologic studies indicated that less than 5% of animals in the mouse colony (all strains) had hemagglutination-inhibition antibodies to Sendai virus.

### Cell Cultures

The UV2237 fibrosarcoma cell line was derived from a UV-induced fibrosarcoma in C3H/HeN mice and adapted to growth *in vitro*[6] and was kindly provided by Dr. M. Kripke of the Frederick Cancer Center. Cultures of mouse embryo cells were prepared by trypsinization of 14–16-day C3H/HeN mouse fetuses as described previously.[7] The embryo cell cultures used in the macrophage-mediated cytotoxicity assays were between the third and the ninth passage *in vitro*, and assays were always done with cultures in the exponential growth phase.

All cells were grown in Eagle's minimal essential medium supplemented with 10% fetal bovine serum, sodium pyruvate, nonessential amino acids, L-glutamine, and two-fold concentrated vitamin solution. The components of this complete medium, designated CMEM, were obtained from Gibco (Grand Island, NY). The same batch of fetal bovine serum was used for the cultivation of cells in all experiments. Cell cultures were incubated at 37 C in a humidified atmosphere containing 5% $CO_2$.

### Macrophages

Populations of inflammatory-tissue macrophages were harvested from subcutaneous inflammatory lesions induced by implanting round glass coverslips, 6 mm in diameter, into the subcutaneous tissue on the dorsum of adult C3H/HeN mice by the use of methods described fully elsewhere.[8] After their removal from subcutaneous tissue, the coverslips, with their adherent cells, were placed in bacterial-grade plastic dishes, 60 mm in diameter, containing CMEM supplemented with penicillin (100 U/ml) and streptomycin (100 μg/ml). Bacterial-grade plastic dishes were used to ensure that when target cells were added in the cytotoxicity assay they attached only to the coverslips and not to areas on the surface of dish that were not covered by the coverslips. After initial plating, the coverslips were maintained in CMEM for 12 hours at 37 C and washed with CMEM to remove nonadherent cells, and the remaining adherent cells were used for the various *in vitro* assays. The density of macrophages on coverslips implanted subcutaneously for 2–4 days was $1.7–2.0 \times 10^5$ cells/sq cm. No significant change in the number of adherent cells was detected with longer implantation. At this density adherent cells cover 80–90% of the surface area of the coverslip. Recent studies [9,10] have shown that the density and total number of macrophages may be of more importance than the macrophage/target-cell ratio in determining the efficiency of macrophage-mediated killing of tumor cells. The density of the macrophage populations recovered on coverslips and the ratio of macrophages to target cells used in this study (see below) are similar to the conditions defined elsewhere [9,10] as optimal for the macrophage-mediated killing of tumor cells.

Peritoneal exudate cells (PECs) were harvested from adult C3H/HeN mice 3 days after intraperitoneal injection of thioglycollate, as described previously.[7] PECs were plated into 60-mm tissue-culture-grade plastic Petri dishes and nonadherent cells decanted 6 hours later [7] to yield a homogeneous adherent cell population (50% of the cells originally plated) that had a typical macrophage structure and phagocytosed carbon particles. PECs were also isolated from adult C3H/HeN mice infected with bacille Calmette Guérin (BCG). Animals were infected intradermally with $5 \times 10^6$ CFU of living *Mycobacterium bovis*, strain BCG (TMC #1029, Phipps strain), in 0.05 ml Middlebrook 7H9 broth (Difco, Detroit, Mich). Six weeks later animals were given injections intraperitoneally of 50 μg purified protein derivative (PPD) (Connaught Medical Research Laboratories, Toronto). Uninfected control animals were given injections of Middlebrook broth without BCG but were challenged six weeks later with PPD. PECs were harvested from PPD-stimulated infected and control animals one day after the PPD injection by peritoneal lavage using 10 ml CMEM-containing heparin (3 U/ml), penicillin (100 U/ml) and streptomycin (100 μg/ml), and adherent macrophage populations recovered as described above. In some experiments PECs were harvested from BCG-infected mice without subsequent PPD stimulation. These animals were given injections intraperitoneally of thioglycollate 6 weeks after infection, and PECs were harvested 3 days later, as described above for PECs from uninfected mice.

### Spleen Cells

Spleen cells were harvested from BCG-infected and control mice after peritoneal lavage had been completed. Spleens were removed aseptically, minced in Hanks' balanced saline solution, and pressed through a 40-mesh stainless steel wire sieve (E-C Apparatus Corp.,

St. Petersburg, Fla). The resulting cell suspensions were then filtered through a glass-wool column and centrifuged at 450*g* for 5 minutes. The cell pellet was resuspended in CMEM and plated into 14-cm glass Petri dishes to remove any contaminating adherent cells that still remained. After incubation for 2 hours at 37 C the nonadherent cells were harvested and the contaminating erythrocytes were lysed by resuspension in cold 0.91% $NH_4Cl$. The surviving cell preparation was composed of >99% lymphocytes, and these were suspended in CMEM at a final concentration of $10^8$ cells/ml for use in experiments.

### *In Vitro* Activation of Macrophages by Lymphokines

Cultures of C3H mouse peritoneal macrophages were activated *in vitro* by incubation for 48 hours at 37 C in lymphokine-rich cell-free culture supernatants harvested from cultures of normal F344 rat lymphocytes stimulated with insoluble Concanavalin A (Con A; Pharmacia, Piscataway, NJ) as described in detail elsewhere [7] and provided by Dr. I. J. Fidler, Frederick Cancer Center, Frederick, Maryland. Cell-free control lymphocyte culture supernatants lacking macrophage activation activity were harvested from F344 rat lymphocytes incubated in culture medium without Con A.[7]

### Assay of Macrophage-Mediated Cytotoxicity *In Vitro*

Macrophage-mediated cytotoxicity was assessed by a radioactive release assay, as described previously.[7] Target cells in the exponential growth phase were incubated for 24 hours in CMEM, supplemented with 0.25 $\mu$Ci/ml [$^{125}$I]-IUDR (New England Nuclear, Boston, Mass; specific activity 200 $\mu$Ci/$\mu$m). The cells were then washed four times with HBSS to remove unbound radioactive label, harvested by trypsinization (0.25% Difco trypsin and 0.05% EDTA for 2 minutes at 37 C), resuspended in CMEM, and plated into dishes 60 mm in diameter containing macrophages to achieve a macrophage/target-cell ratio of between 30:1 and 50:1. Radiolabeled target cells were also plated alone as an additional control group. The macrophage/target-cell cultures were then incubated in CMEM for 5 days at 37 C and refed with CMEM 24 hours after the addition of the target cells. On Day 5 the cultures were washed twice with HBSS to remove nonadherent cells, and the remaining viable, adherent cells were lysed with 1 ml 0.5 N NaOH and washed twice with HBSS. The lysate and the washings were pooled, and their radioactivity was measured using Packard gamma counter. The cytotoxic activity of the macrophages was calculated as follows:

$$\% \text{ cytotoxicity} = 100 \times \frac{\begin{array}{l}\text{cpm [}^{125}\text{I]-IUDR in}\\ \text{target cells cultured}\\ \text{with normal macrophages}\end{array} - \begin{array}{l}\text{cpm [}^{125}\text{I]-IUDR in}\\ \text{target cells cultured}\\ \text{with test macrophages}\end{array}}{\text{cpm [}^{125}\text{I]-IUDR in target cells cultured with normal macrophages}}$$

Spontaneous release of [$^{125}$I]-IUDR from target cells incubated at 37 C in the absence of macrophages for 5 days never exceeded 10% of the total radioactivity incorporated into the cells on Day 1.

### Peroxidase Reactivity

Air-dried coverslips fixed with ethanolic formalin were stained for peroxidase activity by the use of the method of Kaplow.[11]

### Statistical Analysis

Cytotoxicity data were analyzed for statistical significance by the Student *t* test (two-tailed).

## Results

### Morphologic and Cytochemical Properties of Inflammatory Macrophages and MGCs Adhering to Glass Coverslips Implanted in Subcutaneous Tissue

Studies in several laboratories have shown that the cell populations that adhere to glass coverslips implanted subcutaneously are composed exclusively of inflammatory macrophages derived from circulating monocytes.[8] This was confirmed in the present study by the examination of the peroxidase-staining behavior of cells adhering to coverslips implanted subcutaneously in C3H mice. Resident tissue macrophages are peroxidase-negative,[12] but new blood-derived macrophages that accumulate at sites of inflammation are peroxidase-positive.[12] As shown in Table 1, over 95% of the cells that initially adhere to implanted coverslips are peroxidase-positive; but after 4 days the recruitment of peroxidase-positive cells falls rapidly and stops completely after 7 days.

After 2 days 80–90% of the surface area of the coverslip is covered by adherent cells (Figure 1). However, by 4 days the adherent cells begin to fuse to form MGCs (Table 1 and Figure 2). The proportion of MGCs to mononuclear cells increases significantly with longer implantation times (Figure 3), and by 21 days up to 65% of the adherent cells have fused (Table 1).

### The Tumoricidal Properties of Inflammatory Macrophages and MGCs

Inflammatory macrophages recruited onto coverslips during the first 2 days are devoid of spontaneous cytotoxic activity but can be rendered tumoricidal by incubation *in vitro* with lymphokines released by mitogen-stimulated lymphocytes (Table 2). After 4 days the lesions contain inflammatory macrophages that display significant spontaneous cytotoxicity for

Table 1—Peroxidase Reactivity and Multinucleate Giant Cell Formation in Populations of Inflammatory Macrophages Adhering to Coverslips Implanted Subcutaneously in C3H/HeN Mice

| Coverslip implantation time (days) | Peroxidase-positive cells (%)* | Fused cells (%)* |
|---|---|---|
| 2 | 68 | <2 |
| 4 | 96 | 15 |
| 7 | 45 | 38 |
| 10 | 7 | 47 |
| 14 | 0 | 57 |
| 21 | 0 | 65 |
| 28 | 0 | 36 |
| 40 | 0 | 18 |

* Mean values derived from microscopic counts on twelve coverslips obtained from three separate animals. At least 500 cells from each coverslip were examined.

Table 2—*In Vitro* Cytotoxicity of Macrophages Recovered From Subcutaneous Inflammatory Lesions in C3H/HeN Mice

| Coverslip implantation time (days) | Lymphokine treatment | Radioactivity (cpm) in live target cells* C3H/HeN embryo cells | Radioactivity (cpm) in live target cells* UV2237 tumor cells |
|---|---|---|---|
| Nil, target cells alone | | 1759 ± 165† | 3961 ± 382 |
| Nil (C3H/HeN PECs)‡ | −§ | 1834 ± 139 | 3685 ± 276 |
| | +‖ | 1703 ± 192 | 2004 ± 250 (46%)¶# |
| 2 | − | 1682 ± 240 | 3818 ± 319 |
| | + | 1905 ± 175 | 2236 ± 288 (39%)# |
| 4 | − | 1882 ± 168 | 2166 ± 262 (41%)# |
| | + | 1825 ± 207 | 1591 ± 285 (57%)# |
| 7 | − | 1756 ± 193 | 2059 ± 223 (44%)# |
| | + | 1918 ± 216 | 1208 ± 261 (67%)# |
| 10 | − | 1832 ± 158 | 3783 ± 364 |
| | + | 1765 ± 179 | 3511 ± 297 |
| 14 | − | 1527 ± 252 | 3884 ± 285 |
| | + | 1809 ± 176 | 3631 ± 372 |
| 21 | − | 1982 ± 158 | 3796 ± 300 |
| | + | 1792 ± 203 | 3501 ± 318 |

* Target cells were labeled for 24 hours with [$^{125}$I]-IUDR (0.25$\mu$Ci/ml) and plated onto macrophage populations on coverslips at a macrophage/target cell ratio of 50:1. The cultures were maintained in CMEM for 5 days and refed 24 hours after the addition of the target cells and the cell-associated radioactivity remaining in live cells measured as described in Materials and Methods. The results represent mean values derived from three separate experiments. Twelve coverslips were used in each experiment.

† Mean ± SD.

‡ Thioglycollate-induced peritoneal exudate cells incubated *in vitro* in CMEM for 48 hours before use.

§ Macrophages incubated in culture supernatants from normal rat lymphocytes for 48 hours before incubation with target cells.

‖ Macrophages incubated in culture supernatants from Con-A-stimulated rat lymphocyte for 48 hours before incubation with target cells.

¶ Numbers in parentheses represent percentage of cytotoxicity compared with untreated PECs at the same ratio to tumor cells.

# Significant ($P < 0.001$).

tumor cells *in vitro* (Table 2), indicating that they have been activated *in vivo*. Nontumorigenic embryo cells are not destroyed by these cells (Table 2). The tumoricidal activity of these cells can be increased, however, by incubation with lymphokines *in vitro*. Macrophages with spontaneous cytotoxic activity that also respond to lymphokines *in vitro* are present in lesions for up to 7 days. Thereafter, cells with these properties cannot be detected; macrophages harvested on coverslips implanted for 10 days or longer not only lack spontaneous cytotoxic activity but are also refractory to activation *in vitro* by lymphokines.

The cytotoxic activity of inflammatory macrophages from subcutaneous tissue, shown in Table 2, has been calculated in relation to that caused by nonactivated PECs from C3H mice. Although PECs do not represent a

strictly comparable control cell population, they were used as a surrogate to assess the cytotoxic activity of nonactivated macrophages because of the technical difficulty of obtaining nonactivated resident macrophages from subcutaneous tissue in sufficient numbers for these assays.

The results in Table 2 therefore indicate that newly recruited inflammatory macrophages rapidly acquire the ability to kill tumor cells *in vitro*, but they lose this property rapidly. This change is apparently irreversible, and tumoricidal activity cannot be restored by further exposure to lymphokines. The data in Table 2 also indicate that fusion of macrophages to form multinucleate giant cells (MGCs) is not directly responsible for the loss of tumoricidal properties. MGC formation begins at 4 days and is well advanced by 7 days (Table 1) and therefore precedes the start of the decay of tumoricidal activity. Also, macrophages on 10-day coverslips are completely devoid of cytotoxic activity, even though large numbers of unfused cells are present.

#### The Tumoricidal Activity of BCG-Activated Marcophages and MGCs

To determine whether decay of tumoricidal properties and subsequent resistance to activation by lymphokines *in vitro* were peculiar to inflammatory macrophages from subcutaneous tissue, similar experiments were done using PEC populations.

PECs elicited in normal C3H mice by intraperitoneal injection of thioglycollate and activated *in vitro* by incubation *in vitro* for 24 hours with lymphokines from mitogen-stimulated lymphocytes exhibited significant tumoricidal activity (Table 3). However, this property was lost rapidly and was absent after 3–4 days. The reexposure of such cells to lymphokines failed to restore their tumoricidal properties.

Similar results were obtained using PECs activated *in vivo*. These were harvested from BCG-infected animals that had been given intraperitoneal injections of PPD. When assayed for cytotoxic activity immediately after isolation, these cells cause significant destruction of tumor cells (Table 4). However, when cultivated *in vitro* for 3 days or longer before incubation with target cells, they fail to kill tumor cells and are no longer susceptible to activation by lymphokines released by either PPD-stimulated BCG-immune spleen cells or Con-A–stimulated lymphocytes.

Although MCGs are a prominent feature of BCG-induced granulomas, MGCs were not detected in PECs harvested from PPD-stimulated BCG-infected mice or in PECs incubated with BCG *in vitro* for 10 days. However, PECs from BCG-infected mice readily fused to form MGCs when cocultivated *in vitro* with PPD and BCG-immune spleen cells (Table 5). Incubation of similar PEC populations with PPD alone, im-

Table 3—Decay of Tumoricidal Activity in Lymphokine-Activated Peritoneal Exudate Cells From C3H/HeN Mice

| Time in culture (hours)* | Lymphokine treatment† | Second lymphokine treatment‡ | Radioactivity (cpm) in live UV2237 tumor cells (mean ± SD)§ |
|---|---|---|---|
| No macrophages; tumor cells alone | | | 3582 ± 279 |
| 24 | − | − | 3670 ± 342 |
| 24 | + | − | 1677 ± 192 (54%)‖¶ |
| 48 | + | − | 1868 ± 233 (49%)¶ |
| 72 | + | − | 3145 ± 195 (14%)# |
| 96 | + | − | 3496 ± 326 |
| | + | + | 3740 ± 378 |
| 120 | + | − | 3562 ± 277 |
| | + | + | 3817 ± 307 |

* PECs were incubated *in vitro* for the indicated times following isolation from the peritoneal cavity before assay of their cytotoxic activity.

† PECs incubated with lymphokines from Con-A-stimulated lymphocytes for 24 hours following isolation from the peritoneal cavity.

‡ PECs treated with lymphokines as in footnote † were incubated *in vitro* for the indicated times and then incubated with lymphokines for a further 24 hours before assaying their cytotoxic activity.

§ Determined from three separate experiments as described in footnote * in Table 2.

‖ Percentage cytotoxicity compared with control PECs not incubated with lymphokines.

¶ Significant ($P < 0.001$).

# Significant ($P < 0.1$).

mune spleen cells alone or with PPD and nonimmune spleen cells from uninfected animals did not induce MGC formation (results not shown). This suggests that fusion may be facilitated by a factor(s) released by antigen-stimulated immune lymphocytes. This factor may correspond to the so-called "macrophage fusion factor released by sensitized lymphocytes, which has been shown to facilitate fusion *in vitro* of both PECs [13] and alveolar macrophages.[13-16] The reason MGCs occur in PECs from infected animals incubated *in vitro* with PPD-stimulated spleen cells but are absent in PECs harvested from infected animals given injections of PPD *in vivo* is unclear.

Assay of the tumoricidal activity of PEC populations containing MGCs induced by cocultivation *in vitro* with PPD and BCG-immune spleen cells revealed that this property decayed rapidly and that the loss of cytotoxic activity was not reversible by exposure to lymphokines *in vitro* (Table 5). The initial level of cytotoxic activity and the rate of decay of this property are comparable with that seen in normal PECs activated *in vitro* (Table 3) and PECs from BCG-infected animals activated *in vitro* (Table 4). Since MGCs are not present in these latter two situations, it is concluded that MGC formation is not responsible for the changes in

Table 4—Decay of Spontaneous Tumoricidal Activity and Onset of Resistance to *In Vitro* Activation by Lymphokines in Peritoneal Exudate Cells From PPD-Stimulated BCG-Infected Mice*

| Time in culture (hours)† | *In vitro* lymphokine treatment‡ | Radioactivity (cpm) in live UV2237 tumor cells (mean ± SD)§ |
|---|---|---|
| No macrophages; tumor cells alone | | 4286 ± 390 |
| Control PEC from uninfected animals‖ | – | 4015 ± 326 |
| 24 | – | 2119 ± 364 (47%)¶# |
| 48 | – | 2260 ± 274 (44%)# |
| 72 | – | 3565 ± 355 (11%)** |
| | + (PPD) | 3410 ± 327 (15%)†† |
| | + (Con A) | 3529 ± 348 (12%)** |
| 96 | – | 3912 ± 338 |
| | + (PPD) | 4151 ± 386 |
| | + (Con A) | 3863 ± 352 |

* Mice infected 6 weeks earlier with BCG inoculated intraperitoneally with PPD (50 μg) and PECs harvested 24 hours later.

† As in footnote *, Table 3.

‡ PECs were incubated *in vitro* for the indicated times and then incubated for 24 hours with cell-free culture supernatants harvested from PPD-stimulated BCG immune C3H/HeN mouse spleen cells (PPD) or concanavalin-A-stimulated rat lymphocytes (Con A) before assaying their cytotoxic activity.

§ Determined from three separate experiments as described in footnote *, Table 2.

‖ PECs from uninfected mice harvested 3 days after intraperitoneal injection of thioglycollate and incubated *in vitro* for 24 hours before assaying their cytotoxic activity.

¶ Figures in parentheses are percentage of cytotoxicity compared with control PECs from uninfected animals.

# Significant ($P < 0.001$).

** Significant ($P < 0.1$).

macrophage cytotoxicity. Rather, the decay of tumoricidal activity and onset of resistance to activation by lymphokines *in vitro* would appear to affect activated macrophages irrespective of the stimulus responsible for activation. The nature of the activating stimulus and the microenvironment to which macrophages are exposed might be important, however, in determining whether activated macrophages will undergo morphologic conversion to MGCs or simply remain as nontumoricidal mononuclear cells.

## Discussion

The present experiments have shown that macrophages recruited into subcutaneous inflammatory lesions undergo activation and exhibit cytotoxicity for tumor cells while leaving nontumorigenic cells unharmed. Macrophages recruited into these lesions can also be rendered tumoricidal by incubation *in vitro* with lymphokines. These properties are short-lived, however, and with progression of the inflammatory reaction macrophages lose their tumoricidal activity and become resistant to activation by

Table 5—*In Vitro* Cytotoxicity of Peritoneal Exudate Cells from BCG-Infected C3H/HeN Mice Incubated *In Vitro* with PPD and BCG-Immune Spleen Cells*

| Time in culture (hours) | Second lymphokine treatment† | Fused Cells (%)‡ | Radioactivity (cpm) in live UV2237 tumor cells (mean ± SD)§ |
|---|---|---|---|
| No PECs; target cells alone | | | 3452 ± 314 |
| Control PECs from uninfected animals | − | <2 | 3675 ± 355 |
| 24 | − | <2 | 2124 ± 269 (42%)¶# |
| 48 | − | 18 | 2227 ± 310 (39%)# |
| 72 | − | 46 | 3142 ± 304 (14%)** |
| | + | 41 | 3340 ± 339 ( 9%)** |
| 96 | − | 59 | 3762 ± 340 |
| | + | 50 | 3511 ± 382 |

* PECs isolated from PPD-stimulated BCG-infected mice as in footnote *, Table 4, cocultivated *in vitro* for the indicated times with PPD and spleen cells harvested from BCG-infected mice.

† PECs incubated as described in footnote * for the indicated times after which the spleen cells were removed, the PEC cultures washed three times with prewarmed Hanks' balanced saline solution and then incubated for 24 hours with culture supernatants from concanavalin-A-stimulated lymphocytes before assaying their cytotoxic activity.

‡ Mean values derived from microscopic counts on cultures in ten Petri dishes stained *in situ* with May-Grünwald and Giemsa. A minimum of ten microscope fields (×10 objective) were examined from each dish.

§ As in footnote *, Table 2.

‖ As in footnote ¶, Table 4.

# Significant ($P < 0.001$)

** Significant ($P < 0.1$)

lymphokines. A similar rapid decay of tumoricidal properties and the onset of resistance to activation by lymphokines have also been demonstrated in inflammatory macrophages from the peritoneal cavity irrespective of whether activation occurred *in vivo* (PPD-stimulated BCG-infected animals) or *in vitro* (incubation with lymphokines). These results with PEC populations confirm similar observations made recently by Ruco and Meltzer.[17-19] However, the present demonstration that rapid decay of tumoricidal properties also occurs in inflammatory macrophages from subcutaneous tissue suggests that this phenomenon may be a common characteristic of all inflammatory macrophages.

The fusion of macrophages to form MGCs is a prominent feature in the two types of inflammatory macrophage populations examined here. However, this phenomenon is not causally related to the loss of macrophage tumoricidal activity and ability to respond to lymphokines, since unfused cells also exhibit these changes. Rather, the decay of the activated state and reduced responsiveness to lymphokines appear to be a common feature of activated cells, irrespective of the original stimulus responsible for activation. The nature of the activating stimulus and the microenvironment to which activated macrophages are exposed may be important, however, in determining their ability to express other properties and

to undergo morphologic conversion to epitheloid cells or fuse to form MGCs.

The inability of "older" macrophages and MGCs to kill tumor cells and be activated by lymphokines *in vitro* lends support to the view that macrophage-mediated destruction of tumor cells *in vivo* requires the continuous recruitment of new macrophages from the circulation that are susceptible to activation by lymphokines.[4,17] The cytochemical data presented here, showing that recruitment of new, peroxidase-positive macrophages from the circulation into subcutaneous inflammatory lesions declines rapidly after 4 days and ceases after 7–10 days, is also consistent with this interpretation.

The concept that the tumoricidal activity exhibited by macrophage populations *in vivo* reflects the balance between the rate of recruitment of new lymphokine-responsive macrophages from the circulation and the rate of decay of tumoricidal properties in activated cells has several important implications for the role of macrophages in host resistance to tumors. For example, factors that hinder the influx of new, blood-derived macrophages into tumors and/or reduce the turnover of previously recruited macrophages would be expected to reduce the cytotoxic capacity of intratumoral macrophage populations. Of potential importance in this regard are reports showing that certain tumor cells and/or soluble products released by tumor cells can impair monocyte mobilization and decrease their emigration into tumors.[20-23]

The possibility that macrophage antitumor activities *in vivo* are mediated exclusively by newly recruited macrophages is also pertinent to the use of agents such as BCG and *Corynebacterium parvum* in the immunotherapy of neoplastic disease. Intratumoral injection of these agents [24-26] and several other types of "immunopotentiators" [27,28] provokes an intense granulomatous inflammatory reaction that stimulates the infiltration of macrophages, which becomes activated and acquire the ability to kill tumor cells nonspecifically. However, the results presented here suggest that the successful induction of macrophage-mediated antitumor activity by these agents will require that the proportion of activated tumoricidal macrophages (ie, newly recruited cells) consistently exceed the proportion of deactivated macrophages and MGCs. It is therefore of interest that histologic studies of successful tumor regression induced by BCG have emphasized that intratumoral macrophages did not have epithelioid characteristics and that MGCs were absent.[24] However, extensive conversion of subcutaneous inflammatory macrophages to epithelioid cells and MGCs has been reported in adverse complications of BCG immunotherapy in experimental animals [29] and man.[30]

The mechanism(s) responsible for the loss of the tumoricidal phenotype in activated macrophages and their subsequent resistance to reactivation by lymphokines is not known. It has been shown recently, however, that previously activated nontumoricidal PECs that had become refractory to activation by lymphokines *in vitro* could be rendered tumoricidal by treatment *in vitro* with liposomes containing encapsulated lymphokines.[7] This suggests that the failure of these cells to respond to extracellular lymphokines might be due to surface alterations that hinder lymphokine binding. Liposomes, by acting as carrier vehicles whose purpose is to introduce lymphokines into the intracellular environment,[7] are able to circumvent the lesion at the cell surface and induce activation via the action of the released lymphokines on some intracellular target(s).

Although the present results indicate that "older" macrophages and MGCs are nontumoricidal and unresponsive to lymphokines, other studies have shown that these cells continue to express many of the altered enzymic, metabolic, and surface properties that accompany activation by lymphokines.[4,6,8] This dissociation of tumoricidal activity from other features of the activated phenotype thus lends further support to the view that the wide range of phenotypic changes exhibited by activated macrophages are not tightly coupled and can be expressed independently, appearing and decaying at different rates, depending on the stimulus responsible for activation.[4,6,30-35]

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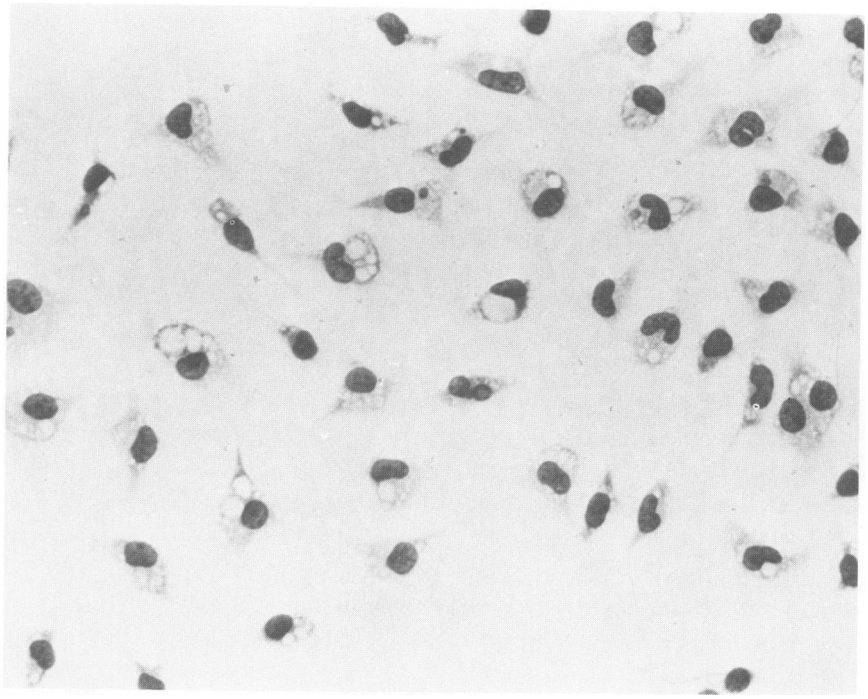

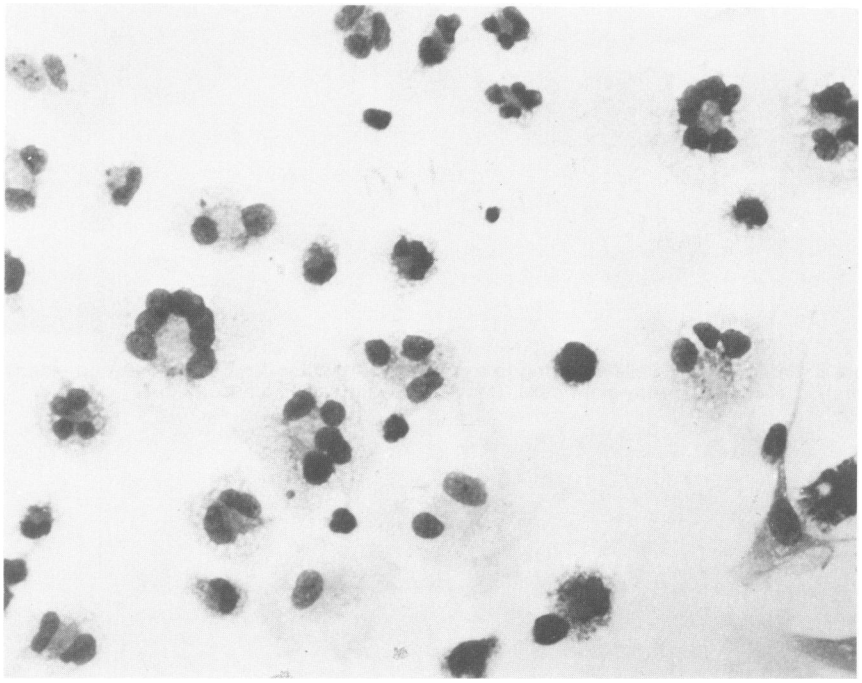

**Figure 1**—Inflammatory tissue macrophages adhering to a glass coverslip implanted in the subcutaneous tissue of a C3H/HeN mouse for 2 days. (May-Grünwald and Giemsa, ×490)
**Figure 2**—Inflammatory macrophages adhering to a coverslip implanted for 4 days, showing the formation of multinucleate giant cells. (May-Grünwald and Giemsa, ×470)

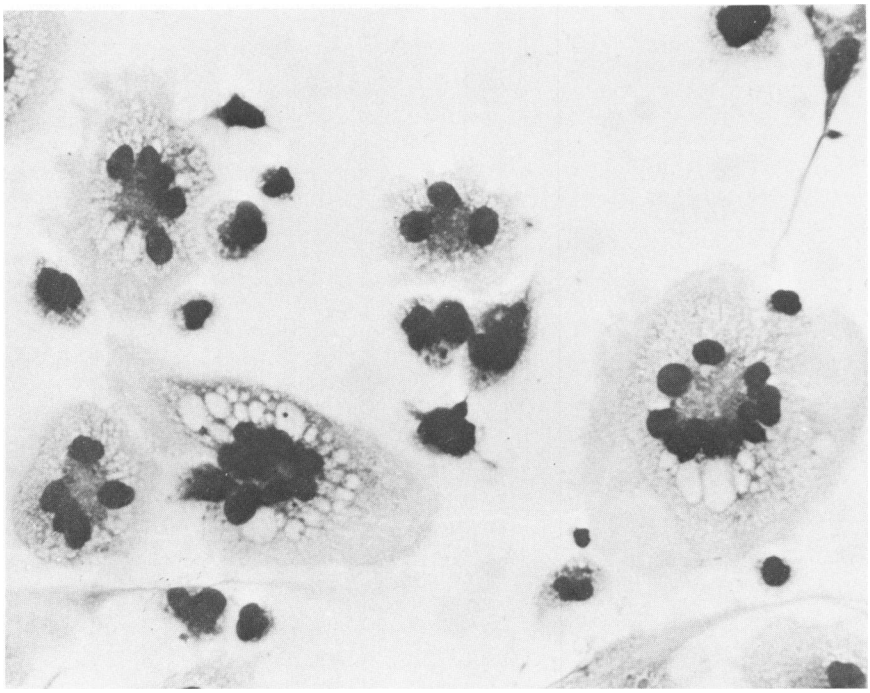

**Figure 3**—Inflammatory cells adhering to a coverslip implanted for 7 days, showing extensive formation of multinucleate giant cells. (May-Grünwald and Giemsa, ×470)